

The Ligninolytic and Biodegradation Potential on Lindane of *Pleurotus Ostreatus* spp

Vicky Dritsa¹, Fotis Rigas²

^{1,2}School of Chemical Engineering, National Technical University of Athens 15700 Athens, Greece

¹vdritsa@central.ntua.gr; ²rigasf@central.ntua.gr

Abstract

Extracellular peroxidases and laccases catalyze the breakdown of organic pollutants via free radical-mediated reactions. In this study, the ligninolytic activities of selected strains of *Pleurotus ostreatus* were determined in a nitrogen-sufficient liquid medium. In order to determine the effect of temperature in ligninolytic activities, the white rot fungi were incubated at different cultivation temperatures (15, 20, 25 and 30°C). The activities of manganese peroxidase and laccase were determined but no lignin peroxidase was detected for all strains. Significant differences were revealed between the strains of the same species. Degenerate primers corresponding to the copper-binding regions in N-terminal domains of known basidiomycetes were used to detect laccase gene. The strain of *Pleurotus ostreatus*, which showed the greatest ligninolytic potential, was used for the biodegradation of lindane in agitated liquid cultures. An orthogonal Central Composite Design of experiments was used to construct second order response surfaces with the fungus growth, final pH and lindane biodegradation as optimization parameters.

Keywords

Biodegradation; Lindane; *Pleurotus Ostreatus*, Laccase; PCR; Central Composite Design

Introduction

Environmental pollutants are a serious worldwide concern because of the hazards they pose to the health of humans, animals and ecosystems. Bioremediation is a promising and cost effective technology. In particular, fungal remediation utilizing white rot fungi has recently received considerable attention for their bioremediation potential, as they have been shown to be effective as degraders of many hazardous environmental pollutants [Dritsa and Rigas, 2011].

Ligninolytic enzymes are produced during the secondary metabolism in nutrient-limited cultures and are capable of degrading persistent pollutants [Barr and Aust, 1994]. They function mainly by the

production of free radicals that are able to attack a wide range of organic molecules. Manganese peroxidase (MnP) and lignin peroxidase (LiP) using H₂O₂ and laccases (polyphenol oxidases) using molecular oxygen are the enzymes responsible for attack on lignin. White rot fungi secrete ligninolytic enzymes during their incubation in liquid cultures or lignocellulosic material. Different white rot species produce various combinations of LiP, MnP and laccase depending on growth substrates [Hattaka, 1994].

Many studies have shown that the ligninolytic system is affected significantly by the nutrients of the medium, such as aromatic compounds, nitrogen or manganese [Faison and Kirk, 1985; Périé and Gold, 1991; Kaal et al., 1995; Levin et al., 2012]. Nevertheless, “and” there are fungi whose production of ligninolytic enzymes is not affected by carbon or nitrogen [Gianfreda et al., 1999]. Specifically, it has been proved that *Phanerochaete chrysosporium* is regulated by nitrogen whereas strains of *Bjerkandera* are not affected [Mester and Field, 1997]. In contrast to the medium composition, the influence of incubation temperature has not been studied extensively. According to Vyas et al. [1994], the incubation temperature affected the appearance, the enzymes patterns, the maximum value and the ratio of LiP and MnP in cultures of *Ph. chrysosporium*. Researchers [Asther et al., 1988; Liebeskind et al., 1990] obtained high productivity of LiP at lower temperatures than the optimum one for the same strain.

Laccases have been extensively studied for potential biotechnological applications. The widespread occurrence of laccases in white rot basidiomycetes, their high redox potential and a requirement for laccase expression for lignin degradation by some white rot fungi strongly implicate white rot fungal laccases in lignin degradation processes. Laccases result in biotechnologically relevant products because

of their ability to oxidize both phenolic and non-phenolic lignin related compounds as well as highly recalcitrant environmental pollutants [Lettera et al., 2011]. Due to the limitations that culture-dependent methods possess, molecular approaches have been reported to characterize fungal strains [D' souza et al., 1996]. The molecular techniques such as the polymerase chain reaction (PCR) amplification of laccase-specific sequences, offer an accurate and rapid means of determining the presence of potential laccase-producing genes [D' souza et al., 1996; Zhao and Kwan, 1999].

In our previous work, an investigation of new strains of white rot fungi, which were isolated from sites in Greece and identified, were screened for their ligninolytic activity using dye decolourisation technique. Among the tested strains, *Pleurotus ostreatus* showed the greatest potential for dye decolourisation and was further used for the degradation of the organochlorine pesticide lindane in liquid cultures. Results obtained from the study showed that *Pl. ostreatus* was successfully selected and its application to bioremediation of lindane contaminated liquid systems is a feasible option due to its removal capabilities [Rigas et al., 2003; Tien and Kirk, 1998].

The aim of this study is the evaluation of the ligninolytic potential of various species of the white rot fungus *Pl. ostreatus*. Since white rot fungi are mesophiles, the effect of temperature and the enzyme production levels were determined at 15, 20, 25 and 30°C. The cultures were cultivated in a nitrogen-sufficient medium which was appropriate for the secretion of the enzymes. The strains were also tested for their growth tolerance towards different concentrations of lindane in agar plates. The most efficient strain of *Pl. ostreatus* (a commercial strain) was studied for the biodegradation of lindane in agitated liquid cultures of a commercial strain of the fungus using the central composite design as a second order experimental design methodology. The investigated factors (variables) were the initial lindane concentration, the nitrogen content (in the form of asparagine), the incubation time and the temperature. The selected optimization parameters (responses) were the fungus growth (biomass), the final pH and the lindane biodegradation. Additionally, polymerase chain reaction (PCR) amplification was used to detect the presence of laccase gene in this fungus using degenerate primers.

Materials and Methods

Microorganisms

The following strains of basidiomycetes from our collection were used in this study: *Pleurotus ostreatus* sp.3, *Pleurotus ostreatus* sp.4, *Pleurotus ostreatus* sp.5 [Rigas et al., 2003].

Culture Media

Cultures were maintained on Potato dextrose Agar (PDA), composed of (g/L): peeled potatoes 200, dextrose 20, and agar 15. The liquid medium for the cultivation was Malt Extract Broth (MEB) containing (g/L) malt extract 17, and mycological peptone 3.

The liquid medium used for enzyme activities, was Kirk's medium composed of KH_2PO_4 0.20, CaCl_2 0.01, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.05, ammonium tartrate 0.22, 2,2-dimethylsuccinic acid 2.90, glucose 5, thiamine 0.1, Tween 80 0.10% v/v, veratryl alcohol 1.5 mM, trace elements (10mL). The trace elements composed of (mg/L): MnSO_4 33, $\text{Fe}_2(\text{SO}_4)_3$ 50, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 43, $\text{CuSO}_4 \cdot 7\text{H}_2\text{O}$ 80, H_2MoO_4 50 [Tien and Kirk, 1998].

Media were sterilized by autoclaving at 121°C for 20 minutes.

Enzyme Assays

Inocula (1-cm diameter) were cut from grown PDA cultures and transferred in 2% malt extract broth (50 mL in 125 mL Erlenmeyer flasks). The flasks were incubated for 7 days at 25°C under stationary conditions. The cultures were homogenized using a blender and 10-mL aliquots were added to 90 mL Kirk's medium (in 250 mL Erlenmeyer flasks). The cultures were further incubated in four temperatures, 15, 20, 25 and 30°C. Samples (1 mL) were aseptically removed from the liquid cultures at various times and tested for enzyme activities. Three replicates were performed for each strain. The culture filtrates were used for the estimation of manganese peroxidase, lignin peroxidase and laccase.

Laccase activity was determined spectrophotometrically. The method is based on the formation of the quinone form of syringaldazine at 25°C when incubated with the phenoloxidase preparation [Szklarz et al., 1989].

Lignin peroxidase activity was determined according to the method of Tien and Kirk [Tien and Kirk, 1998]. Lignin peroxidase catalyses the oxidation of veratryl alcohol to veratraldehyde by H_2O_2 .

Manganese peroxidase activity was measured by the method based on the formation of quinone of syrigaldazine at 525 nm. The enzymatic activities were expressed in U/L [Rigas et al., 2005].

Isolation of DNA

Four mycelial plugs (1 cm in diameter) of each fungus strain grown on PDA dishes were inoculated into MEB (50 mL in 125 mL Erlenmeyer flasks). The cultures were incubated for 7 days at 25°C, under static conditions. The mycelia were harvested from the liquid medium by filtration and were frozen at -20°C. DNA was isolated using CTAB extraction [Gardes and Bruns, 1993].

PCR Amplification

Genomic DNA isolated from each fungus was used as template for PCR amplification. The PCR reaction contained: 5 µL PCR buffer, 3 µL MgCl₂, 2 µL genomic DNA, 1 µL of each nucleotide triphosphate, 1 µL of each primer, 0.5 µL Taq polymerase adjusted to 50 µL sterilized deionized distilled water. Primer sequences for laccase genes have been published previously as follows: degenerate primers I and II [14, 15] Primer I: 5'-CAY TGG CAY GGN TTY TTY CA and primer II: 5'-RTG RCT RTG RTA CCA RAA NGT. The temperature programme was carried out in a DNA thermal cycler. An initial cycle of denaturation (5 min at 94°C), annealing (2 min at 54°C), and extension (5 min at 72°C) was followed by 35 cycles of denaturation (1 min at 94°C), annealing (2 min at 54°C), and extension (5 min at 72°C) and then by a final incubation (10 min at 72°C). In each experiment, we used negative controls to test the contamination of the reagents and the template DNA.

PCR products were analyzed by electrophoresis on 1.2% agarose gels stained with ethidium bromide in 1XTBE buffer. A 10-µL sample of each reaction and 2-µL gel loading dye were loaded into lanes on the gel. The 100-bp DNA size marker was run in a separate lane. The PCR products were electrophoresed for 2 hours at 90V. Gels were visualized with UV light and photographed. The resulting images were digitized and sizes of the bands were determined with Gel Analyzer software (Biosure).

Tolerance Test

Fungal strains were tested for their growth rate in solid media, containing the organochlorine pesticide lindane. Lindane has been extensively used in the past for the control of agricultural and medical pests. It

poses serious environmental hazards due to its toxicity and bioaccumulation arising from its persistence in water, soil and sediments. The media used, was PDA and four different concentrations of lindane (5, 10, 15, 20 mg/L). Inocula (1 cm diameter) were cut with a cork borer from fully-grown PDA cultures and transferred onto Petri dishes. The growth rate was measured in mm/d. All the plates were incubated at 25°C in darkness until the mycelia grew and reached the edge of the Petri dish (90 mm diameter). Five replicates were performed for each strain.

Degradation of Lindane in Liquid Cultures

Each 125-mL Erlenmeyer flask contained 45 mL of Basal Medium under different concentrations of asparagine and 5 mL homogenised mycelium. The flasks were agitated (90 rpm) and aseptically incubated in the respective temperatures according to the statistical design. Two replicates were performed for each experiment and one control consisted of uninoculated flask. At the end of each experiment, the fungal biomass was separated from the medium by filtration. Filtrate, biomass and flask were extracted separately for lindane. Biomass was first dried in air, then 2 mL of hexane was added and the suspension mixed vigorously for 10 min. In the flask, 1 mL of hexane was added and was shaken for 2 min. For the filtrate extraction, 0.5 mL sample was diluted in 1.5 mL deionized water and mixed with 10 mL hexane for 10 min. The upper phase was collected and stored with the extracts of flask and biomass in darkness at 4°C until further analysis. For all the procedures, the chemicals and solvents used were of the highest purity available. For the analysis of the samples, a gas chromatograph (Shimadzu GC-17A) equipped with an Electron Capture Detector and 30 m×0.32 mm Optima-5 column. The injector, detector and column temperatures were 270, 280 and 60°C, respectively. The N₂ flow rate was programmed at 50 mL per min, and 1 µL samples were injected each time for the analysis.

A central composite design was applied with four design factors, namely the initial lindane concentration (X₁), the nitrogen content supplied from asparagine (X₂), the incubation time (X₃) and the incubation temperature (X₄). The most important key factors in the degradation of xenobiotics are the tolerance level, time, temperature, fungal nutrition, and particularly the need for nitrogen. Nutrition plays a crucial role in the onset and intensity of secondary metabolism because limiting the supply of an essential nutrient is

an effective mean of restricting growth and can have specific metabolic and regulatory effect. Therefore, it is prerequisite to design a proper culture medium in an efficient biodegradation process the sensitivity and the degradation ability of a fungus are influenced by the concentration of the pollutant. Temperature and time are also factors controlling biodegradation. The coded levels and the natural values of these factors set in the statistical experiment are shown in Table 1.

TABLE 1 CODED (IN BOLD) AND NATURAL VALUES OF DESIGN FACTORS

Design factors	-1.483	-1	0	+1	+1.483
X ₁ :Lindane content (mg/L)	2.03	3	5	7	7.97
X ₂ : Nitrogen content (g/L)	0.11	0.4	1	1.6	1.89
X ₃ : Time (days)	3.55	5	8	11	12.45
X ₄ : Temperature (°C)	15.6	18	23	28	30.4

Results and Discussion

Enzyme Activities

Apart from the already known factors that restrict the degradation of xenobiotic compounds by white rot fungi in liquid media and soil (e.g. limited bioavailability, hydrophobic properties, and production of H₂O₂), the levels of ligninolytic enzymes proved to be an important factor when aiming at higher biodegradation rates. Therefore, the expression and production of the ligninolytic enzymes should be taken into consideration in the development of bioremediation techniques. It has been shown that enzyme activities are sensitive to aeration and agitation [Novotny et al., 2004]. Therefore, the cultures were incubated statically in order to be evaluated at their optimum values. The combination of manganese peroxidase and laccase was predominant for the tested strains. The negative test for lignin peroxidase implies that either these fungi do not produce LiP in significant levels or LiP production requires different conditions [Pelaez et al., 1995] as in the case of *Trametes versicolor*, *Bjerkandera adusta*, known as LiP producers [Waldner et al., 1998; Kirk and Farrell, 1987]. Therefore, the production was dependent on the strain or the cultivation conditions. The maximum activities and the day of their expression for all strains are listed in Table 2.

Values are reported as means and standard deviations of three replicates. Laccase activities did not vary significantly among the strains at 15° C. The highest laccase activity under the tested conditions was obtained by *Pl. ostreatus* sp.3, attaining 6.15 U/L after eleven days of growth. Manganese peroxidase activities were higher compared to the laccase ones under the same conditions. The same strain had the highest production of MnP, 15.66 U/L after 12-day incubation time. The production of laccase was highest at 20°C for all strains. The highest levels of laccase were achieved by *Pl. ostreatus* sp.3 producing 21.6 U/L, respectively. MnP peroxidase levels at the same conditions, ranged in lower levels with regard to laccase. *Pl. ostreatus* sp.3 was individuated, expressing the highest MnP level (13.21 U/L). Laccase and MnP were expressed by all fungi at 25°C and *Pl. ostreatus* sp.3 showed high production of the two enzymes. The maximum MnP and laccase activities were expressed by *Pl. ostreatus* sp.3 at 30°C (FIG.1)

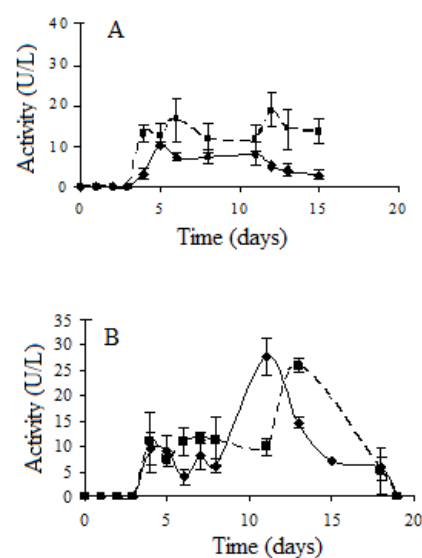


FIG. 1 TIME COURSE OF LACCASE AND MANGANESE PEROXIDASE PRODUCTION BY *PLEUROTUS OSTREATUS* SP.3 AT 25°C (A) AND 30°C (B). (—■—) MnP ACTIVITY, (—♦—) LAC ACTIVITY

PCR Amplification

Laccases of white rot fungi are copper-containing glycoproteins which catalyse the one-electron oxidation of various substrates and the concurrent four-electron reduction of oxygen to water. Till now, the genes for numerous laccases have been cloned and the sequences have been registered in electronic libraries. According to available nucleotide sequences of laccase genes from basidiomycetes [D' souza et al., 1996], the PCR performed with this primer pair is

TABLE 2 MAXIMUM LACCASE AND MANGANESE PEROXIDASE ACTIVITIES FOR THE TESTED STRAINS
AT 15, 20, 25 AND 30°C (MEAN $\pm$ S.D.)

Temperature	Enzyme activities (U/L)\n(Expression day)					
	<i>Pl. ostreatus sp.3</i>		<i>Pl. ostreatus sp.4</i>		<i>Pl. ostreatus sp.5</i>	
	Laccase	MnP	Laccase	MnP	Laccase	MnP
15	6.15 $\pm$ 1.41 (11)	15.66 $\pm$ 2.0 (12)	5.53 $\pm$ 1.22 (14)	13.09 $\pm$ 2.83 (4)	4.59 $\pm$ 1.03 (13)	11.21 $\pm$ 2.3 (10)
20	21.61 $\pm$ 1.16 (4)	13.21 $\pm$ 2.08 (12)	10.19 $\pm$ 1.56 (9)	6.56 $\pm$ 0.76 (4)	9.69 $\pm$ 1.12 (10)	5.58 $\pm$ 1.02 (12)
25	10.31 $\pm$ 0.66 (5)	18.71 $\pm$ 4.10 (12)	8.15 $\pm$ 0.7 (12)	14.37 $\pm$ 2.89 (11)	6.61 $\pm$ 1.03 (13)	8.45 $\pm$ 1.57 (12)
30	27.81 $\pm$ 3.62 (11)	25.96 $\pm$ 1.44 (13)	4.31 $\pm$ 0.7 (4)	8.18 $\pm$ 0.40 (6)	3.59 $\pm$ 0.65 (8)	7.32 $\pm$ 2.11 (10)

expected to produce fragments of approximately 200 bp (FIG.2). For the strains of basidiomycetes tested, the PCR products were analysed by agarose gel electrophoresis. All the strains of *Pl. ostreatus* showed one DNA band of 200 bp which was attributed to laccase gene fragments. Conclusively, the PCR strategy based on the use of primers corresponding to specific domains of laccase sequences is an indispensable approach to screening white-rot fungi for the presence of laccase genes [Zhao and Kwan,1999; Luis et al., 2004].

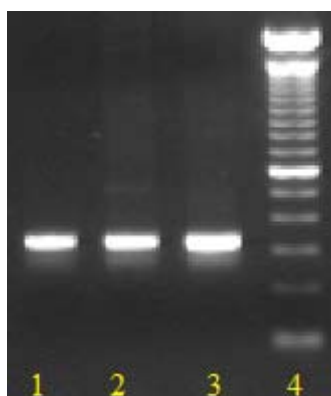


FIG. 2 ELECTROPHORESIS OF PCR PRODUCTS. LANES : 1 *PLEUROTUS OSTREATUS SP. 3*, 2 *PLEUROTUS OSTREATUS SP. 4*, 3 *PLEUROTUS OSTREATUS SP. 5*, 4 DNA LADDER 100 BP

Tolerance Test

The strains were inoculated on Petri dishes in PDA medium to test their tolerance against four different concentrations of lindane (5, 10, 15 and 20 ppm). The growth rates decreased with increasing concentration of lindane for all strains (TABLE 3). Significant differences of the growth rate were found among the *Pl. ostreatus* strains tested.

TABLE 3 GROWTH RATES OF FUNGAL STRAINS ON PETRI DISHES IN PDA MEDIUM FOR FOUR CONCENTRATIONS OF LINDANE

Fungal strains	Growth rate (mm/d) (Mean $\pm$ S.D.) Lindane concentration (ppm)				
	0	5	10	15	20
<i>Pleurotus ostreatus sp.3</i>	5.33 $\pm$ 0.22	4.50 $\pm$ 0.07	4.03 $\pm$ 0.35	3.52 $\pm$ 0.37	2.45 $\pm$ 0.19
<i>Pleurotus ostreatus sp.4</i>	1.78 $\pm$ 0.11	1.14 $\pm$ 0.07	1.20 $\pm$ 0.09	0.72 $\pm$ 0.05	0.66 $\pm$ 0.07
<i>Pleurotus ostreatus sp.5</i>	1.65 $\pm$ 0.15	1.33 $\pm$ 0.21	1.03 $\pm$ 0.11	1.01 $\pm$ 0.12	0.77 $\pm$ 0.22

Treatment of the growth vs. time with least squares in the linear region resulted in acceptable correlation factors ranging from 0.95 to 0.98 in all cases. *Pleurotus ostreatus sp.3* followed a linear decrease of growth rate with lindane concentration, with R^2 correlation coefficient equal to 0.97, as it is shown in FIG. 3.

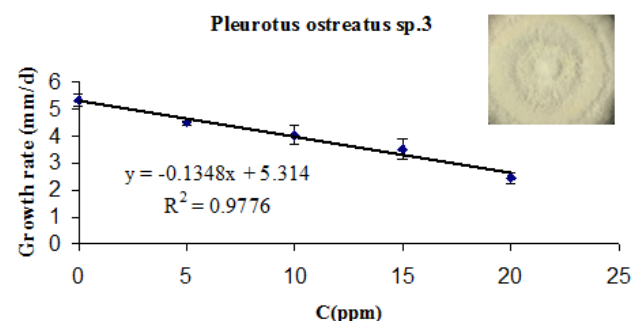


FIG. 3 GROWTH RATES OF *PLEUROTUS OSTREATUS SP.3* ON PETRI DISHES IN PDA MEDIUM FOR FIVE CONCENTRATIONS OF LINDANE (0, 5, 10, 15 AND 20 PPM). THE RADIAL GROWTH OF THE FUNGUS IS SHOWN IN THE PHOTO.

Degradation of Lindane in Liquid Cultures

The optimization parameters selected were the fungus growth (biomass) and various expressions of the lindane biodegradation, as follows:

$Y_1 = M_f$	Fungus growth (mg)
$Y_2 = M_{l0} - M_l$	Biodegraded mass of lindane (μg)
$Y_3 = (M_{l0} - M_l) / M_{l0}$	Extent of biodegradation relative to initial lindane mass (mg/g)
$Y_4 = (M_{l0} - M_l) / M_{l0} M_f$	Extent of biodegradation relative to initial lindane mass and to final biomass (mg/g/g)
$Y_5 = \text{pH}$	Final pH

The models obtained after running the 52 trials of the central composite design and excluding the insignificant effects are the following:

$$Y_1 = 90.0 + 38.5 X_3 - 31.5 X_4 - 37.0 X_1 X_3$$

$$Y_2 = 0.0981 + 0.0325 X_1 - 0.0301 X_1 X_4 + 0.0256 X_2 X_4 - 0.0252 X_4^2$$

$$Y_3 = 0.328 + 0.103 X_3 + 0.114 X_2 X_4$$

$$Y_4 = 9.10 - 2.96 X_1 - 3.30 X_2^2 + 2.87 X_2 X_3$$

$$Y_5 = 5.418 - 0.442 X_2 - 0.458 X_3 + 0.558 X_1^2 + 0.869 X_3 X_4$$

All these models were tested with the lack-of-fit criterion and were found adequate with the exception of the model for Y_4 , which failed at the 95% confidence level (p -value = 0.02). This model can be improved with the addition of some more replicates or can be considered adequate at a somewhat lower confidence level.

Since all these second order models do not show straightaway the effects of the factors on the optimization parameters, for the sake of three-dimensional visualizations only two factors at a time against an optimization parameter can be plotted. Thus, two sample response surfaces were constructed and are shown in FIG. 4.

The selected fungus (*Pl. ostreatus* sp. 3) showed a maximum growth of 275 mg at the factors combination of the lowest level of lindane content (2.03 mg/L), intermediate nitrogen content (0.51 g/L), the highest level of time (12.45 d) and the lowest level of temperature (15.6°C). The maximum biodegradation of lindane, expressed as the extent of biodegradation relative to initial lindane mass and to final biomass was found equal to 14.6 mg/g/g

(degraded lindane / initial lindane / biomass), at a region close to the fungus growth maximum, namely 2.03 mg/L lindane, 1.32 g/L nitrogen content, 12.04 days and 15.7°C. Thus, it was verified that the biodegradation of lindane increases with the fungus growth and the produced degradative enzymes measured in this work. At the same region the optimum for pH (2.5) was determined.

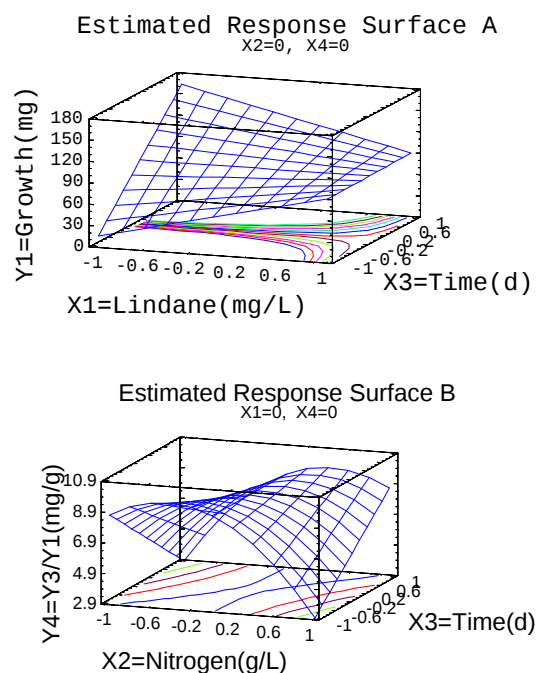


FIG. 4 RESPONSE SURFACES FOR Y_1 =GROWTH AGAINST X_1 =LINDANE CONTENT AND X_3 =TIME (SURFACE A) AND FOR Y_4 = BIODEGRADATION RELATIVE TO INITIAL LINDANE MASS AND FINAL BIOMASS AGAINST X_2 =NITROGEN CONTENT AND X_3 =TIME (SURFACE B)

Conclusions

In this study, three strains of *Pleurotus ostreatus* were tested in order to estimate their ligninolytic potential. The enzyme activities showed that the combination of laccase and manganese peroxidase prevailed in all strains. It was confirmed that the incubation temperature affected the expression and the pattern of the ligninolytic enzymes. Significant differences were revealed between laccase and manganese peroxidase activities, implying that the enzyme production depends not only on the strain but also on the cultivation conditions. *Pleurotus ostreatus* sp.3 showed the highest laccase and MnP activity at 30°C and was individuated from the other two strains. PCR amplification products gave the expected ~200 bp band in all basidiomycetes. The three strains of *Pl. ostreatus* displayed a single band with the specific pair of primers. Certain isolates were highly tolerant to lindane in agar medium and grew up to a

concentration of 20 mg/L lindane. The growth rates differ significantly along the four concentrations of lindane for all strains. Significant differences, revealed in the expression and the levels of the ligninolytic enzymes of the same species, are in agreement with previous studies, implying that the enzyme production of fungi depends not only on the strain but also on the cultivation conditions. Conclusively, the response surface methodology selected (central composite design) proved to be suitable for performing bioremediation studies in complex biological systems where the growth of microorganisms in a hostile medium, the production of degradative enzymes by the microorganism, the toxicity of the pollutant to the microorganism, the heterogeneity of the system's components and other factors do not permit a straightforward study and, consequently, the black-box of Cybernetics appears redeeming.

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